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# Class-switched marginal zone B cells in spleen have relatively low numbers of somatic mutations

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ABSTRACT

The vast majority of rodent splenic marginal zone (MZ)-B cells are naive IgM$^+$ cells. A small fraction of these MZ-B cells carry mutated V-genes, and represent IgM$^+$ memory MZ-B cells. Here we reveal further heterogeneity of B cells with a MZ-B cell phenotype, by providing evidence for the existence of class-switched memory MZ-B cells in the rat. In essence, we observed IGHV5 encoded C$\gamma$ transcripts, among FACS-purified MZ-B cells, defined as HIS24$^{low}$HIS57$^{bright}$ cells. Furthermore, we found that most IgG encoding transcripts are mutated. There is no significant difference in IGHV5 repertoire and subclass usage of these IgG encoding transcripts collected from B cells with a MZ-B cell phenotype and B cells with a follicular (FO) B cell phenotype. However, the IGHV5 genes encoding for IgG antibodies of MZ-B cells exhibited significantly fewer mutations, compared to those with a FO-B cell phenotype. In one rat we found a clonally related set of IgG encoding sequences, of which one was derived from the MZ-B cell fraction and the other from the FO-B cell fraction. We speculate that these two subpopulations of class-switched B cells are both descendants from naive FO-B cells and are generated in germinal centers. Class-switched memory cells with a MZ-B cell phenotype may provide the animal with a population of IgG memory cells that can respond rapidly to blood-borne pathogens.



## 1. Introduction

The marginal zone (MZ) represents a distinct anatomical B cell compartment in the spleen located at the outer areas of the white pulp, at the border of the red pulp (for review see e.g. Steiniger et al., 2006). The circulatory system of the spleen ensures an intimate contact of blood and cells of the MZ. Most of the cells in this compartment are B cells, but macrophages and dendritic cells (and in humans also CD4$^+$ T cells) are also present. MZ-B cells have unique characteristics (for reviews see e.g. Martin and Kearney, 2002; Pillai et al., 2005; Weill et al., 2009). In rodents the vast majority of MZ-B cells expresses high levels of IgM and low levels of IgD (IgM$^{hi}$IgD$^{lo}$) in combination with high levels of CD21 and low levels of CD23 (CD21$^{hi}$CD23$^{lo}$) (Oliver et al., 1997). This unique phenotype distinguishes them from the majority population of mature, naive B cells, i.e. follicular (FO) B cells, which are IgM$^{lo}$IgD$^{hi}$CD21$^{lo}$CD23$^{hi}$. Rat FO-B cells can also be defined as mature (i.e. CD90$^-$ (Kroese et al., 1995)) small-sized, HIS24$^{high}$HIS57$^{neg/low}$ B cells whereas MZ-B cells are slightly larger cells and can be distinguished as CD90$^-$HIS24$^{low}$HIS57$^{high}$ cells (Dammers et al., 1999; Kroese et al., 1990, 1995). Importantly, MZ-B cells also have different functional characteristics, such as their pre-activated status and their proliferative and stimulatory requirements (Oliver et al., 1997, 1999). Rodent MZ-B cells appear to be biased towards T cell-independent (TI-2) immune responses against micro-organism-derived polysaccharide antigens (Guinamard et al., 2000; Martin et al., 2001; Vinuesa et al., 2003). These properties in combination with their topographical localization in spleen, allow them to respond rapidly to blood-borne pathogens by the generation of massive numbers of antibody secreting cells during the first few days after infection (Martin et al., 2001).

MZ-B cells are a heterogeneous population of cells, and comprise both naive and memory cells. In rats and mice, the majority (up to

*Abbreviations:* FO, follicular; GC, germinal center; IGHV, Ig heavy chain V region genes; IGHD, Ig heavy chain D region genes; IGHJ, Ig heavy chain J region genes; HEL, hen egg lysozyme; MZ, marginal zone; TI-2 antigen, T cell independent type 2 antigen.

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80%) of MZ-B cells are naive cells which express germline encoded V region of the Ig genes (Dammers et al., 2000; Makowska et al., 1999). Occurrence of memory B cells in the MZ was first demonstrated by Liu et al. (1988), showing the appearance of hapten-binding, $IgM^+$ memory cells with a MZ-B cell phenotype in the MZ of spleens from immunized rats. Hapten-binding MZ-B cells were also demonstrated by flow-cytometry and/or immunohistology in several subsequent studies in immunized normal and Ig-gene targeted mice (Gatto et al., 2004, 2007; Obukhanych and Nussenzweig, 2006; Pape et al., 2003; Phan et al., 2005). In rodents, memory cells constitute a minority MZ-B cell population. Up to 20% of the rodent MZ-B cells might be IgM memory B cells as indicated by the presence of mutated Ig H chain V gene (IGHV) trancripts encoding for IgM antibodies (IGHV-Cμ transcripts) among purified $sIgM^+$ MZ-B cells (Dammers et al., 2000; Makowska et al., 1999). There are some data that suggest that, in addition to these unswitched IgM-expressing memory MZ-B cells, also some class-switched (memory) B cells are found among the MZ-B cell population in rodent spleens after immunization (Gatto et al., 2004; Liu et al., 1988; Obukhanych and Nussenzweig, 2006; Pape et al., 2003). For example, Gatto et al. (2004) observed the presence of $IgG^+$ phage (Qβ)-specific B cells with a MZ phenotype (i.e. $CD21^{hi}CD23^{low}$ B cells), up to 21 days upon immunization of normal mice. Whether these class switched MZ-B cells were indeed "classical" memory cells with their characteristic mutated high affinity BCR's was, however, not investigated. The presence of mutated, antigen-specific (memory) MZ-B cells was subsequently demonstrated in mice 12 days after immunization with viral particles (Gatto et al., 2007). The isotype of these cells was, however, not known, but the authors speculated that these mutated MZ-B cell sequences were derived from class-switched cells. In humans, a relative large proportion (30%) of the MZ-B cells (defined as $CD21^+CD23^-CD27^+$ cells) appear to express IgG (Ettinger et al., 2007); the mutational status of these isotype-switched IGHV genes is also not known.

Thus, although both $IgM^+$ and $IgG^+$ memory cells appear to be present among the pool of MZ-B cells in both rodents and humans, direct evidence for presence of MZ-B cells with mutated IgG encoding genes is currently lacking. Furthermore, the origin of the $IgG^+$ memory MZ-B cells is enigmatic. This prompted us to analyze in detail the nucleotide sequences of IgG encoding (IGHV-Cγ) transcripts from purified rat MZ-B cells, defined in a sIg independent fashion. We show that indeed naturally occurring (i.e. without deliberate antigenic stimulation) MZ-B cells express mutated IGHV genes encoding for IgG antibodies. The repertoire of the MZ-B cell derived IgG encoding transcripts does not differ from that obtained from class-switched B cells with a FO-B cell phenotype, albeit that MZ-B cell derived IgG encoding transcripts exhibit lower numbers of mutations.

## 2. Materials and methods

### 2.1. Animals

Male PVG rats were purchased from Harlan (Horst, The Netherlands) at the age of 6–8 weeks. Animals were maintained until use under clean conventional conditions at the central animal facility of the University Medical Center Groningen. Experiments were approved by the Animal Ethics Committee of the University of Groningen.

### 2.2. Flow-cytometry

Spleens were taken from 4.5 to 8 months old animals. Single-cell suspensions were prepared from spleen and labeled with mAb as described previously (Dammers et al., 1999). Briefly, spleen cell suspensions from 4 animals were stained for flow-cytometry with the following two sets of mouse monoclonal antibodies: FITC conjugated anti-rat IgM (HIS40; eBioscience, San Diego, CA, USA) and biotinylated anti-rat IgD (MaRD3; AbD Serotec, Oxford, UK) or FITC anti-rat MZ-B cell marker (HIS57; BD Pharmingen, San Diego, CA, USA; Dammers et al., 1999) and biotinylated anti-rat CD45R (HIS24; Ebioscience). Biotinylated mAb were revealed with streptavidin conjugated to the tandem fluorochrome PE-Cy5.5 (Ebioscience). The two sets of antibodies were used in combination with a mixture of PE conjugated anti-rat TCRαβ (R73; eBioscience); TCRγδ (V65; eBioscience), CD90/Thy1.1 (HIS51; eBioscience) and CD161a/NKR-P1a (10/78; BD Pharmingen). The PE channel was used as a "Dump" channel; only PE negative ($Dump^-$) cells were sorted. Herewith, we were able to exclude immature B cells (i.e. CD90 positive B cells: Kroese et al., 1995), T cells and NK cells from our sorts. Cell analysis and cell sortings were performed on a MoFlo flow cytometer (Cytomation, Ft Collins, CO). Dead cell, plasma cell, monocyte/macrophage, and erythrocyte contamination was excluded from sorting by using forward and side scatter profiles. Sorted cells were collected in sterile FACS tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) containing 500 μl of newborn calf serum (PAA laboratories GmbH, Pasching, Austria). At least one million cells per B cell subset were sorted. B cell subsets were obtained with >95% purity. FlowJo software (Tree Star, San Carlos, CA) was used for flow cytometry data analysis.

### 2.3. Molecular cloning of rearranged IGHV5-Cγ transcripts

Total RNA was extracted from sorted cells using the Absolutely RNA Miniprep kit (Stratagene, La Jolla, CA, USA) according to instructions of the manufacturer. Briefly, sorted cells were pelleted by $300 \times g$ centrifugation for 10 min at 4 °C and then resuspended in a total volume of 350 μl lysis buffer containing β-mercaptoethanol (Stratagene). First strand cDNA was synthesized using an oligo-$(dT)_{12-18}$ primer (Invitrogen, Breda, The Netherlands) and SuperScript™ II reverse transcriptase (200 U/μl; Invitrogen) as described in the manufacturer's protocol. Rearranged immunoglobulin IGHV5-Cγ transcripts were amplified in a 50 μl reaction mixture, containing 2 μl cDNA and 0.6 pmol/μl IGHV5 (PC7183) family specific primer (5′-CTTAGTGCAGCCTGGAAGGT-3′; Dammers et al., 2000), 0.6 pmol/μl universal Cγ constant region primer (5′-GACAGGGATCCAGAGTTCCA-3′) and 2.5 U Taq DNA Polymerase (Invitrogen). The universal Cγ region primer was designed on the basis of a conserved sequence found in exon 1 of all rat IgG subclasses. To assess the amount and quality of the cDNA, PCR was also performed for β-actin, using β-actin specific primers as described by Stoel et al. (2008). The PCR program for amplification of IGHV5-Cγ transcripts and β-actin consisted of 35 cycles of 30 s at 94 °C (2 min in first cycle), 1 min at 58 °C and 1 min at 72 °C, respectively. This program was followed by an additional incubation period of 25 min at 72 °C to allow extension of all IGHV5-Cγ products. The quality and size of the PCR products was evaluated by agarose gel electrophoresis. PCR products were subsequently cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was isolated from randomly picked colonies with the Nucleospin Plasmid QuickPure kit (Clontech, Mountain View, CA, USA). Plasmids containing an insert of approximately 600 bp were sequenced in both directions at our local sequence facility (Department of Pathology and Laboratory Medicine, Division of Medical Biology, University Medical Center Groningen, Groningen, The Netherlands). Sequence processing was performed using ClustalW from the European Molecular Biology Laboratory and Chromas software (Digital River GmbH, Cologne, Germany).

### 2.4. Analysis of IGHV5-Cγ transcripts

Analysis of IGHV5-Cγ transcripts (accession numbers pending), was carried out as described previously (Dammers et al., 2000). Briefly, IGHV region sequences were compiled according to the format of the International Immunogenetics (IMGT) database (http://imgt.cines.fr) (Lefranc et al., 1999). Rearranged IGHV5 genes were compared to the 28 previously established PVG germline IGHV5 genes (Dammers and Kroese, 2001) and to two newly identified germline IGHV5 genes from PVG rat (PC-39 and PC-41). PC-39 and PC-41 were established on the basis the following GenBank database accession numbers: AJ286206, AJ286170, and AJ286224 (PC-39); AJ286269, AJ286226, and AJ286210 (PC-41). IGHV5 gene sequences were considered to be germline when two or more independently sampled, rearranged or genomic, IGHV5 gene sequence(s) share 100% identity upon alignment. Germline IGHD and IGHJ gene sequences were taken from the IMGT database. IgG subclasses were determined by aligning the Cγ nucleotide sequence from the IGHV5-Cγ transcripts to the NCBI rat genome database using the BLASTN program (http://www.ncbi.nlm.nih.gov). The accession numbers for the C region genes encoding for rat IgG subclasses are: IgG2a, BC088240; IgG2b, M28671; IgG1, BC095846; and IgG2c, X07189.

### 2.5. Statistical analysis

Statistical analysis of the data was performed using SPSS 16 software (SPSS Inc. Chicago, IL, USA). IGHV5 sequences displaying 100% identity were considered to be derived from a single B cell and counted only once for statistical analysis. We used Fisher's exact test for comparison of IGHV, IGHD, IGHJ gene usage, the subclass distribution of the expressed Cγ regions and the number of mutations between the different groups. In all statistical tests we considered a $P$-value < 0.05 to be significant. The number of mutations was determined by counting the number of nucleotide mismatches in comparison with each IGHV5 gene sequence to its closest germline counterpart. Possible differences in H-CDR3 length between different groups were tested with the Mann–Whitney test. The R/S mutation ratio is the quotient of replacement (R) to silent (S) mutations. R/S mutation ratios are calculated separately for H-CDR1 and 2 and the H-FR chain. The theoretical expected (inherent) R/S mutation ratio is the quotient of total possible R to total possible S mutations in the germline gene, as described by Chang and Casali (1994). The probability that an excess or scarcity of R mutations in the H-CDR or the H-FR results solely from chance is negated by the significantly low probability values ($P$ < 0.05) calculated according to the Binomial distribution.

## 3. Results

### 3.1. Mutated IGHV5-Cγ transcripts are found among B cells with a MZ-B cell phenotype

Classical memory B cells are class-switched and carry somatically hypermutated IGHV genes (see e.g. Tangye and Tarlinton, 2009). In order to see whether class-switched memory B cells with a MZ-B cell phenotype exist, we first analyzed the presence of IgG encoding (IGHV-Cγ) transcripts among FACS-purified B cell subsets defined in a sIg-independent fashion. As shown in Fig. 1, FO-B cells are mature (i.e. CD90$^-$; Kroese et al., 1995) small-sized, HIS24$^{high}$HIS57$^{neg/low}$ B cells whereas MZ-B cells are slightly larger cells and are defined as CD90$^-$HIS24$^{low}$HIS57$^{high}$ cells (Dammers et al., 1999; Kroese et al., 1990). Plasma cells are not included in these two B cell fractions, since they lack expression of HIS24-determinant (CD45R) (Kroese et al., 1987). We first looked for the presence of IGHV-Cγ transcripts encoding for IGHV5 (PC7183) family genes using RT-PCR. For comparison, we also analyzed IGHV5-Cγ transcripts from B cells with a FO-B cell phenotype (CD90$^-$HIS24$^{high}$HIS57$^{neg/low}$) and from a fraction of cells that should include classical, class-switched, memory B cells, i.e. IgM$^-$IgD$^-$ (non-T, non-NK) cells. This IgM$^-$IgD$^-$ fraction of cells comprises class-switched B cells with a FO-B cell and a MZ-B cell phenotype. In all four rats analyzed, we found expression of IGHV5-Cγ transcripts in all three B cell fractions (i.e. MZ-B, FO-B, and IgM$^-$IgD$^-$ cell phenotypes). These observations indicate that class-switched B cells are present both among the sorted MZ-B cells and FO-B cells. Since presence of mutations is another hallmark of (most) classical memory cells, IGHV5-Cγ transcripts from the three B cell subsets were subsequently cloned and sequenced. As shown in Table 1, nearly all sequences were uniquely and productively rearranged. Only very few 100% identical IGHV5-Cγ sequences were found (three sets in the FO-B cell fraction and one set in the IgM$^-$IgD$^-$ cell fraction). These sequences were counted only once in our further analysis, since we could not rule out the possibility that they originate from the same cell. In total, we obtained 33 unique productive IGHV5-Cγ transcripts from the MZ-B cell fraction (HIS24$^{low}$HIS57$^{high}$), 27 from the FO-B cell fraction (HIS24$^{high}$HIS57$^{neg/low}$) and 37 from the class-switched B cell fraction (IgM$^-$IgD$^-$). Alignment of the constant region of these transcripts to known constant regions of rat revealed that all sequences were indeed encoded by Cγ genes (Table 1), and that the V-regions of the transcripts were encoded by IGHV5 genes. Nearly all sequences reveal somatic mutations upon comparison to the nearest germline sequence of PVG rats. Since *Taq* errors might be responsible for 1–2 mutations per sequence, we considered only sequences with more than 2 mutations as truly mutated (Dammers et al., 2000). Using this criterion, at least 80% of the IGHV5-Cγ sequences of all three fractions display somatic mutations (Table 1). Because of the presence of Cγ transcripts and mutated IGHV5 genes, we provide evidence for the existence of classical, class-switched, memory B cells in the phenotypically defined MZ-B cell and FO-B cell compartments.

### 3.2. IGHV5-Cγ transcripts from B cells with a MZ-B cell phenotype exhibit fewer somatic mutations compared to B cells with a FO-B cell phenotype

The average number of mutations and the mutation frequency of the IGHV5-Cγ transcripts from B cells with a MZ-B cell phenotype appeared to be lower than that obtained from B cells with a FO-B cell phenotype: $7 \pm 4.9$ (i.e. $2.9 \pm 2.07\%$) (mean ± s.d.) vs $10 \pm 8.0$ mutations (i.e. $4.3 \pm 3.36\%$) per IGHV gene, respectively (Table 1). Dividing the IGHV5-Cγ transcripts into four categories regarding the number of mutations, i.e. unmutated (0–2 mutations), low (3–5 mutations), intermediate (6–10 mutations) and high (>10 mutations), revealed that the number of mutations in IGHV5-Cγ transcripts from sorted MZ-B cells differ statistically significantly from transcripts from sorted FO-B cells (Fisher's exact test, $P = 0.004$). As we show in Fig. 2, transcripts from B cells with a MZ-B cell phenotype are enriched in the category with an intermediate number of mutations whereas most sequences from B cells with a FO-B cell phenotype and IgM$^-$IgD$^-$ cells are found in the category of a high number of mutations.

### 3.3. Class-switched memory type MZ-B cells have a similar IgG subclass distribution as their FO-B cell counterpart

There are four IgG subclasses in rat: IgG1, IgG2a, IgG2b and IgG2c. Based upon sequence identity, we were able to establish the IgG subclasses of the IGHV5-Cγ trancripts. The constant region primer used to amplify IGHV5-Cγ trancripts was located in the first

**Table 1**
Sequence analysis of IGHV5-C$\gamma$ transcripts from MZ-B cells, FO-B cells and IgM$^-$IgD$^-$ cells from adult rat spleen.

| Clone | Rat | IGHV5 member | IGHD member | IGHJ member | Subclass | H-CDR3 | | Mutations[b] | | R/S mutation rate FR | | | R/S mutation rate H-CDR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | N[a] | Amino acids | F[c] | N[d] | Obs[e] | Exp[f] | P[g] | Obs[e] | Exp[f] | P[g] |
| A. sequences from MZ-B cells | | | | | | | | | | | | | | | |
| MZ1 | 1 | PC-15 | IGHD1-7 | IGHJ4 | IgG2b | 16 | TTRTIAAISTSYVLDA | 2.53% | 6 | 1/3 | 3.04 | 0.03689 | 1/1 | 3.63 | 0.40130 |
| MZ2 | 1 | PC-3 | IGHD5-1 | IGHJ1 | IgG1/IgG2a | 13 | ARPKNWEGWCFDF | 0.42% | 1 | 0/0 | 3.1 | 0.39710 | 0/1 | 4.75 | 0.83270 |
| MZ3 | 1 | PC-4 | IGHD1-7 | IGHJ2 | IgG2c | 14 | ARHDGMMVVSPFGY | 1.27% | 3 | 3/0 | 3.07 | 0.21760 | 0/0 | 4.44 | 0.58160 |
| MZ4 | 1 | PC-39 | IGHD1-7 | IGHJ3 | IgG2c | 16 | ARPGVTTVVTWNWFAY | 0.42% | 1 | 1/0 | 3.07 | 0.60140 | 0/0 | 4.44 | 0.83470 |
| MZ5 | 1 | PC-29 | IGHD1-7 | IGHJ4 | IgG1/IgG2a | 15 | SKDYYYDASYYVMDA | 1.69% | 4 | 2/0 | 2.99 | 0.34710 | 2/0 | 3.21 | 0.10230 |
| MZ6 | 1 | PC-34 | IGHD1-2 | IGHJ3 | IgG2c | 12 | TREDPDITWFSY | 4.64% | 11 | 4/1 | 3.13 | 0.06680 | 3/3 | 3.79 | 0.16800 |
| MZ7 | 1 | PC-5 | IGHD1-2 | IGHJ1 | IgG2c | 10 | ARKDSWFFDF | 0.00% | 0 | 0/0 | 2.99 | 1.00000 | 0/0 | 4.48 | 1.00000 |
| MZ8 | 1 | PC-1 | IGHD5-1 | IGHJ2 | IgG2b | 9 | ASLNWELDY | 1.27% | 3 | 1/1 | 3.11 | 0.28480 | 1/0 | 3.48 | 0.33520 |
| MZ9 | 1 | PC-4 | IGHD1-7 | IGHJ3 | IgG2c | 14 | ARHSGMVVITPFAY | 0.84% | 2 | 2/0 | 3.07 | 0.36170 | 0/0 | 4.44 | 0.69670 |
| MZ10 | 1 | PC-5 | IGHD1-6 | IGHJ2 | IgG1/IgG2a | 11 | ASESYGGLFDY | 0.84% | 2 | 0/1 | 2.99 | 0.16210 | 1/0 | 4.48 | 0.27630 |
| MZ11 | 2 | PC-5 | IGHD4-1 | IGHJ2 | IgG2b | 10 | ARAIRDYFDY | 4.64% | 11 | 4/3 | 2.99 | 0.07209 | 2/2 | 4.48 | 0.29570 |
| MZ12 | 2 | PC-4 | IGHD1-6 | IGHJ2 | IgG1/IgG2a | 13 | ARHDYGGYSELGY | 3.80% | 9 | 4/1 | 3.07 | 0.16580 | 3/1 | 4.44 | 0.12830 |
| MZ13 | 2 | PC-29 | IGHD1-3 | IGHJ3 | IgG2b | 15 | AKASYYFSSYNWFTY | 2.95% | 7 | 2/1 | 2.99 | 0.07930 | 3/1 | 3.21 | 0.06591 |
| MZ14 | 2 | PC-28 | IGHD1-7 | IGHJ3 | IgG2b | 9 | ARYDAPLTY | 4.22% | 10 | 5/1 | 2.99 | 0.20280 | 3/1 | 3.55 | 0.14200 |
| MZ15 | 2 | PC-6 | IGHD1-6 | IGHJ2 | IgG2b | 8 | TTGGYGDY | 3.38% | 8 | 5/0 | 3.21 | 0.28030 | 3/0 | 3.79 | 0.09627 |
| MZ16 | 2 | PC-31 | IGHD1-6 | IGHJ4 | IgG1/IgG2a | 12 | ARHPNYGPLMDA | 2.11% | 5 | 0/1 | 3.01 | 0.01039 | 2/2 | 3.86 | 0.15290 |
| MZ17[j] | 2 | PC-39 | IGHD5-1 | IGHJ2 | IgG2c | 8 | TAGTGFEY | 2.53% | 6 | 4/1 | 3.07 | 0.31180 | 1/0 | 4.44 | 0.40190 |
| MZ18[k] | 3 | PC-5 | IGHD1-6 | IGHJ2 | IgG1/IgG2a | 12 | ARHEEGAGYFDY | 3.38% | 8 | 3/3 | 2.99 | 0.12630 | 2/0 | 4.48 | 0.25910 |
| MZ19[j] | 3 | PC-11 | IGHD1-7 | IGHJ2 | IgG1/IgG2a | 11 | ARQRGSYYPDY | 6.33% | 15 | 8/3 | 3.04 | 0.17710 | 2/2 | 4.56 | 0.27320 |
| MZ20 | 3 | PC-36 | IGHD4-4 | IGHJ4 | IgG1/IgG2a | 11 | ARLGIAGVMDA | 3.38% | 8 | 4/3 | 3.03 | 0.23250 | 1/0 | 3.96 | 0.37630 |
| MZ21 | 3 | PC-28 | IGHD1-4 | IGHJ3 | IgG2b | 13 | STLRYYGYNPFGY | 3.38% | 8 | 3/1 | 2.99 | 0.12630 | 4/0 | 3.55 | 0.02193 |
| MZ22 | 3 | PC-22 | IGHD1-5 | IGHJ2 | IgG2b | 10 | ARHFITTFDY | 1.27% | 3 | 1/0 | 2.96 | 0.29190 | 2/0 | 3.86 | 0.06515 |
| MZ23[l] | 3 | PC-4 | IGHD1-1 | IGHJ2 | IgG2b | 15 | ARHLGATTVVTPFDY | 2.53% | 6 | 2/0 | 3.07 | 0.13690 | 3/1 | 4.44 | 0.05254 |
| MZ24 | 3 | PC-1 | IGHD1-4 | IGHJ1 | IgG2c | 16 | ARTYYGYNPHYWYFDF | 0.00% | 0 | 0/0 | 3.11 | 1.00000 | 0/0 | 3.48 | 1.00000 |
| MZ25 | 3 | PC-5 | IGHD1-7 | IGHJ4 | IgG2b | 20 | ARPKPVTIFSDGSLGFVLDA | 10.55% | 25 | 12/5 | 2.99 | 0.07848 | 7/1 | 4.48 | 0.06307 |
| MZ26 | 4 | PC-3 | IGHD3-3 | IGHJ2 | IgG2b | 11 | VRAEYLHYFDY | 3.80% | 9 | 4/0 | 3.1 | 0.16440 | 4/1 | 4.75 | 0.03952 |
| MZ27 | 4 | PC-26 | IGHD3-4 | IGHJ3 | IgG1/IgG2a | 8 | AGDVPFTY | 3.80% | 9 | 2/3 | 3.16 | 0.01951 | 4/0 | 4.48 | 0.03831 |
| MZ28 | 4 | PC-22 | No D present | IGHJ2 | IgG2b | 8 | ARHPYFDY | 3.80% | 9 | 5/0 | 2.96 | 0.25250 | 4/0 | 3.86 | 0.03511 |
| MZ29 | 4 | PC-29 | IGHD1-6 | IGHJ2 | IgG2b | 11 | ARQEPLRGFDY | 3.80% | 9 | 3/2 | 2.99 | 0.07629 | 4/0 | 3.21 | 0.03099 |
| MZ30 | 4 | PC-36 | IGHD1-3 | IGHJ1 | IgG2b | 13 | TRFGYSRYWYFDF | 4.64% | 11 | 4/4 | 3.03 | 0.07036 | 2/1 | 3.96 | 0.29400 |
| MZ31 | 4 | PC-29 | IGHD1-6 | IGHJ2 | IgG1/IgG2a | 13 | VKDGINNGGPFDY | 3.80% | 9 | 6/0 | 2.99 | 0.24910 | 2/1 | 3.21 | 0.26540 |
| MZ32 | 4 | PC-5 | IGHD5-1 | IGHJ2 | IgG1/IgG2a | 12 | ARETTGDYYFDY | 3.38% | 8 | 3/1 | 2.99 | 0.12630 | 4/0 | 4.48 | 0.02550 |
| MZ33 | 4 | PC-27 | IGHD1-4 | IGHJ2 | IgG2b | 12 | ARRELGITLFDY | 1.69% | 4 | 0/0 | 3.16 | 0.02412 | 4/0 | 3,93 | 0,00068 |
| B. Sequences from FO-B cells | | | | | | | | | | | | | | | |
| FO1 | 1 | PC-22 | IGHD1-3 | IGHJ2 | IgG2b | 10 | ARRAYSSYPY | 0.42% | 1 | 0/0 | 2.96 | 0.40410 | 1/0 | 3.86 | 0.16090 |
| FO2 | 1 | PC-3 | IGHD4-1 | IGHJ2 | IgG2b | 13 | AAGNSGQRGFFDY | 0.84% | 2 | 1/0 | 3.1 | 0.47880 | 1/0 | 4.75 | 0.27860 |
| FO3 | 1 | PC-34 | IGHD1-4 | IGHJ3 | IgG1/IgG2a | 13 | TRLSPGITRPFAY | 4.64% | 11 | 4/4 | 3.13 | 0.06680 | 3/0 | 3.79 | 0.16800 |
| FO4 | 1 | PC-31 | IGHD1-6 | IGHJ1 | IgG1/IgG2a | 13 | ARQGEGITWYFDF | 2.11% | 5 | 3/0 | 3.01 | 0.34560 | 2/0 | 3.86 | 0.15290 |
| FO5 | 1 | PC-41 | IGHD1-7 | IGHJ3 | IgG1/IgG2a | 15 | ARHQDGSYYYSWFAY | 0.42% | 1 | 0/1 | 3.2 | 0.39260 | 0/0 | 4.44 | 0.83470 |
| FO6 | 1 | PC-24 | IGHD3-1 | IGHJ4 | IgG1/IgG2a | 14 | TTDANYPGTYIMDA | 3.80% | 9 | 3/1 | 3.11 | 0.07190 | 2/3 | 4.56 | 0.27850 |
| FO7 | 1 | PC-27 | IGHD1-7 | IGHJ2 | IgG2b | 10 | ARGWSGTLDY | 2.53% | 6 | 2/1 | 3.16 | 0.13280 | 3/0 | 3.93 | 0.04962 |
| FO8 | 1 | PC-28 | IGHD1-7 | IGHJ3 | IgG2c | 10 | TRGWNNWFPY | 2.53% | 6 | 1/1 | 2.99 | 0.03793 | 3/1 | 3.55 | 0.04709 |
| FO9 | 1 | PC-4 | IGHD4-4 | IGHJ4 | IgG1/IgG2a | 16 | ARHPPNLLLGGYVMDA | 2.11% | 5 | 3/0 | 3.07 | 0.34560 | 2/0 | 4.44 | 0.15890 |
| FO10 | 1 | PC-5 | IGHD1-1 | IGHJ3 | IgG1/IgG2a | 13 | ARRDMDPWDWFAY | 2.11% | 5 | 1/2 | 2.99 | 0.07850 | 2/0 | 4.48 | 0.15930 |
| FO11 | 2 | PC-1 | IGHD1-6 | IGHJ2 | IgG2b | 7 | ATSGGSY | 5.49% | 13 | 5/3 | 3.11 | 0.06310 | 5/0 | 3.48 | 0.03157 |
| FO12 | 2 | PC-1 | IGHD1-6 | IGHJ3 | IgG1/IgG2a | 14 | AREGDMAAGAWFAY | 0.84% | 2 | 0/1 | 3.11 | 0.15740 | 0/1 | 3.48 | 0.71000 |
| FO13 | 2 | PC-34 | IGHD3-2 | IGHJ2 | IgG1/IgG2a | 14 | SRGGGFIAAIYFDY | 4.64% | 11 | 4/0 | 3.13 | 0.06680 | 5/2 | 3.79 | 0.01713 |
| FO14[i] | 2 | PC-1 | IGHD1-7 | IGHJ1 | IgG2c | 14 | ARHRTMVVITPFDF | 5.49% | 13 | 5/1 | 3.11 | 0.06310 | 5/2 | 3.48 | 0.03157 |
| FO15 | 2 | PC-5 | IGHD1-4 | IGHJ2 | IgG1/IgG2a | 9 | VRHGPGYKF | 7.59% | 18 | 6/5 | 2.99 | 0.01531 | 6/1 | 4.48 | 0.04358 |
| FO16 | 2 | PC-14 | IGHD1-7 | IGHJ3 | IgG2b | 19 | AKKGTYFYHGSYDVGWFAY | 8.02% | 19 | 8/5 | 2.96 | 0.05618 | 5/1 | 4.15 | 0.11110 |
| FO17[i,j] | 2 | PC-5 | IGHD3-4 | IGHJ3 | IgG2b | 11 | AKASTANWFAY | 8.02% | 19 | 10/3 | 2.99 | 0.14870 | 6/0 | 4.48 | 0.05315 |
| FO18 | 2 | PC-11 | IGHD1-7 | IGHJ2 | IgG1/IgG2a | 18 | TRGDPIYYYDGSYGYFDY | 15.61% | 37 | 17/9 | 3.04 | 0.02963 | 9/2 | 4.56 | 0.07403 |
| FO19 | 3 | PC-26 | IGHD5-1 | IGHJ1 | IgG1/IgG2a | 13 | ARLLNWELWYFDF | 6.75% | 16 | 7/3 | 3.16 | 0.07865 | 6/0 | 4.48 | 0.02700 |
| FO20 | 3 | PC-1 | IGHD1-6 | IGHJ2 | IgG1/IgG2a | 10 | AKDKNYGGFY | 6.75% | 16 | 7/5 | 3.11 | 0.08098 | 3/1 | 3.48 | 0.23560 |
| FO21 | 3 | PC-2 | No D present | IGHJ2 | IgG2b | 5 | TTRDY | 0.42% | 1 | 0/0 | 3.03 | 0.40040 | 1/0 | 3.48 | 0.15740 |
| FO22 | 3 | PC-23 | IGHD1-6 | IGHJ2 | IgG2a/IgG1 | 9 | TTTEALFDY | 4.64% | 11 | 5/2 | 3.11 | 0.14390 | 4/0 | 3.55 | 0.06171 |
| FO23 | 3 | PC-34 | IGHD5-1 | IGHJ2 | IgG2c | 10 | TRENWLPGYN | 1.27% | 3 | 1/0 | 3.13 | 0.28380 | 2/0 | 3.79 | 0.06471 |
| FO24[i] | 3 | PC-4 | IGHD1-6 | IGHJ1 | IgG1/IgG2a | 18 | TRHPPGEGFSDHSWYFDF | 6.75% | 16 | 9/2 | 3.07 | 0.18820 | 3/2 | 4.44 | 0.24150 |
| FO25 | 3 | PC-26 | IGHD1-7 | IGHJ3 | IgG1/IgG2a | 18 | ARLAYYYDGSYYYGRFAY | 5.06% | 12 | 5/3 | 3.16 | 0.09550 | 3/1 | 4.48 | 0.19580 |
| FO26[k] | 3 | PC-5 | IGHD1-6 | IGHJ2 | IgG1/IgG2a | 12 | ARHEEGAGYFDY | 5.06% | 12 | 7/2 | 2.99 | 0.22750 | 2/1 | 4.48 | 0.29610 |
| FO27 | 3 | PC-24 | IGHD1-4 | IGHJ2 | IgG2b | 11 | TRDPGITGFDY | 2.11% | 5 | 3/1 | 3.11 | 0.34560 | 1/0 | 4.56 | 0.40160 |
| C. Sequences from IgM$^-$IgD$^-$ cells | | | | | | | | | | | | | | | |
| CM1 | 1 | PC-29 | IGHD1-5 | IGHJ2 | IgG1/IgG2a[h] | 10 | AKDGKQLFDS | 4.64% | 11 | 3/4 | 2.99 | 0.02430 | 4/0 | 3.21 | 0.05803 |
| CM2 | 1 | PC-35 | IGHD5-1 | IGHJ2 | IgG1/IgG2a | 12 | AKRLPGYHYFDY | 3.38% | 8 | 4/2 | 3.10 | 0.23000 | 2/0 | 3.79 | 0.25220 |
| CM3 | 1 | PC-24 | IGHD4-4 | IGHJ3 | IgG1/IgG2a | 4 | GMGQ | 5.06% | 12 | 2/4 | 3.11 | 0.00232 | 6/0 | 4.56 | 0.00653 |
| CM4[i] | 1 | PC-27 | IGHD1-7 | IGHJ2 | IgG1/IgG2a | 14 | ASPSWHYTGHAGDY | 2.11% | 5 | 4/1 | 3.16 | 0.26560 | 0/0 | 3.93 | 0.41460 |
| CM5 | 1 | PC-31 | IGHD1-4 | IGHJ2 | IgG2b | 15 | ARHPLFRYNSLGFDY | 0.84% | 2 | 1/0 | 3.01 | 0.48050 | 1/0 | 3.86 | 0.27000 |
| CM6 | 1 | PC-5 | IGHD3-4 | IGHJ3 | IgG1/IgG2a | 11 | ARHEVGGWFAY | 0.00% | 0 | 0/0 | 2.99 | 1.00000 | 0/0 | 4.48 | 1.00000 |
| CM7[j] | 1 | PC-1 | IGHD4-1 | IGHJ3 | IgG1/IgG2a | 16 | TTDLIIRGRDPNWFVY | 5.91% | 14 | 4/3 | 3.11 | 0.01280 | 7/0 | 3.48 | 0.00247 |
| CM8 | 1 | PC-24 | IGHD1-6 | IGHJ1 | IgG1/IgG2a | 17 | TTGGGSSYIYPGWYFDF | 0.42% | 1 | 1/0 | 3.11 | 0.60330 | 0/0 | 4.56 | 0.83390 |
| CM9 | 1 | PC-24 | IGHD1-6 | IGHJ2 | IgG1/IgG2a | 12 | TTETYGGSYFDY | 5.91% | 14 | 3/3 | 3.11 | 0.00306 | 8/0 | 4.56 | 0.00059 |
| CM10 | 2 | PC-34 | IGHD1-8 | IGHJ2 | IgG2b | 14 | TSGDYDGYYPAGDY | 2.95% | 7 | 2/3 | 3.13 | 0.07435 | 2/0 | 3.79 | 0.22520 |

Table 1 (*Continued*)

| Clone | Rat | IGHV5 member | IGHD member | IGHJ member | Subclass | H-CDR3 | | Mutations[b] | | R/S mutation rate FR | | | R/S mutation rate H-CDR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | N[a] | Amino acids | F[c] | N[d] | Obs[e] | Exp[f] | P[g] | Obs[e] | Exp[f] | P[g] |
| CM11 | 2 | PC-14 | IGHD5-1 | IGHJ2 | IgG2b | 12 | ARHGPSNSLFDY | 0.84% | 2 | 1/1 | 3.04 | 0.48000 | 0/0 | 4.15 | 0.70020 |
| CM12 | 2 | PC-15 | IGHD1-8 | IGHJ2 | IgG2b | 12 | ARHPIVEDYFDY | 0.84% | 2 | 1/1 | 3.04 | 0.48000 | 0/0 | 3.63 | 0.70760 |
| CM13 | 2 | PC-4 | IGHD4-2 | IGHJ3 | IgG1/IgG2a | 12 | TRRGKVGDWFAY | 1.27% | 3 | 1/0 | 3.07 | 0.28660 | 0/2 | 4.44 | 0.58160 |
| CM14 | 2 | PC-34 | IGHD1-3 | IGHJ2 | IgG1/IgG2a | 13 | ARQPFYSGYPFDY | 1.69% | 4 | 3/0 | 3.13 | 0.34930 | 1/0 | 3.79 | 0.37960 |
| CM15 | 2 | PC-22 | IGHD1-6 | IGHJ4 | IgG2b | 14 | TRSGRLTTKGVMDA | 2.53% | 6 | 2/0 | 2.96 | 0.14210 | 2/2 | 3.86 | 0.19250 |
| CM16 | 2 | PC-14 | IGHD1-7 | IGHJ2 | IgG2b | 11 | SRHDFYGFPED | 4.64% | 11 | 5/2 | 3.04 | 0.14720 | 4/0 | 4.15 | 0.06725 |
| CM17 | 2 | PC-34 | IGHD3-2 | IGHJ1 | IgG1/IgG2a | 13 | SRQGSHQNWYFDF | 2.95% | 7 | 3/3 | 3.13 | 0.18930 | 1/0 | 3.79 | 0.39340 |
| CM18 | 2 | PC-2 | IGHD1-3 | IGHJ2 | IgG1/IgG2a | 16 | TRDRSKFDYSGYYFDY | 2.11% | 5 | 1/1 | 3.03 | 0.07706 | 3/0 | 3.48 | 0.02767 |
| CM19 | 3 | PC-8 | IGHD1-7 | IGHJ2 | IgG2c | 14 | TRQRFTMMPVDFDY | 1.27% | 3 | 1/0 | 3.14 | 0.28340 | 2/0 | 4.00 | 0.06600 |
| CM20 | 3 | PC-1 | IGHD1-6 | IGHJ2 | IgG2b | 13 | ARPPYGGYGLLDY | 7.17% | 17 | 9/4 | 3.11 | 0.15790 | 4/0 | 3.48 | 0.15760 |
| CM21 | 3 | PC-5 | IGHD1-7 | IGHJ1 | IgG1/IgG2a | 20 | ARQTYFYDGSYYYRYWYFDF | 2.11% | 5 | 2/0 | 2.99 | 0.23290 | 3/0 | 4.48 | 0.03160 |
| CM22[l] | 3 | PC-4 | IGHD1-1 | IGHJ2 | IgG2c | 15 | ARHLGATTVVTPFDY | 5.91% | 14 | 5/1 | 3.07 | 0.03998 | 7/1 | 4.44 | 0.00327 |
| CM23 | 3 | PC-25 | IGHD1-8 | IGHJ3 | IgG1/IgG2a | 14 | ARSTITAISNWFAY | 4.64% | 11 | 7/2 | 2.96 | 0.23480 | 2/0 | 3.86 | 0.29360 |
| CM24 | 3 | PC-27 | IGHD1-4 | IGHJ2 | IgG2b | 13 | ARHEYRYNYYFDY | 0.84% | 2 | 0/2 | 3.16 | 0.15530 | 0/0 | 3.93 | 0.70320 |
| CM25 | 3 | PC-6 | IGHD1-6 | IGHJ1 | IgG2b | 15 | AAYGGYSELAWYFDL | 2.53% | 6 | 2/0 | 3.21 | 0.13080 | 3/1 | 3.79 | 0.04876 |
| CM26 | 3 | PC-8 | IGHD1-2 | IGHJ2 | IgG1/IgG2a | 13 | ARRGILWVPYFDY | 5.06% | 12 | 7/1 | 3.14 | 0.22600 | 4/0 | 4.00 | 0.08294 |
| CM27 | 3 | PC-41 | No D present | IGHJ3 | IgG2b | 7 | ARHWFAY | 0.42% | 1 | 0/0 | 3.20 | 0.39260 | 0/1 | 4.44 | 0.83470 |
| CM28 | 3 | PC-5 | IGHD1-6 | IGHJ2 | IgG1/IgG2a | 20 | ARQERFYSTFSYSGLDFFDY | 5.06% | 12 | 3/3 | 2.99 | 0.01304 | 6/0 | 4.48 | 0.00643 |
| CM29 | 4 | PC-15 | IGHD1-1 | IGHJ2 | IgG1/IgG2a | 17 | SRYPIIYYGLLSRPFDY | 4.22% | 10 | 5/3 | 3.04 | 0.20070 | 1/1 | 3.63 | 0.33490 |
| CM30 | 4 | PC-5 | IGHD1-5 | IGHJ2 | IgG2b | 11 | AREELDTYYGY | 1.27% | 3 | 1/0 | 2.99 | 0.29050 | 2/0 | 4.48 | 0.06863 |
| CM31 | 4 | PC-1 | IGHD1-2 | IGHJ3 | IgG2b | 12 | ARPGHSGFWFAY | 3.38% | 8 | 4/0 | 3.11 | 0.22970 | 3/1 | 3.48 | 0.09270 |
| CM32 | 4 | PC-5 | IGHD3-1 | IGHJ2 | IgG1/IgG2a | 11 | ARRPTVSPFDY | 4.64% | 11 | 4/1 | 2.99 | 0.07209 | 4/2 | 4.48 | 0.06985 |
| CM33 | 4 | PC-29 | IGHD3-3 | IGHJ3 | IgG1/IgG2a | 9 | TKVSNCFGY | 1.69% | 4 | 1/0 | 2.99 | 0.15600 | 3/0 | 3.21 | 0.01246 |
| CM34 | 4 | PC-39 | IGHD1-3 | IGHJ4 | IgG2b | 15 | ARHGQYSSYDDVMDV | 3.80% | 9 | 4/1 | 3.07 | 0.16580 | 4/0 | 4.44 | 0.03812 |
| CM35 | 4 | PC-5 | IGHD1-4 | IGHJ2 | IgG1/IgG2a | 9 | TPVGYGHNY | 7.59% | 18 | 9/5 | 2.99 | 0.13100 | 3/1 | 4.48 | 0.24520 |
| CM36 | 4 | PC-15 | IGHD1-7 | IGHJ2 | IgG2b | 14 | TRDPSYYYSNSLDY | 0.84% | 2 | 2/0 | 3.04 | 0.35990 | 0/0 | 3.63 | 0.70760 |
| CM37 | 4 | PC-24 | IGHD1-3 | IGHJ2 | IgG1/IgG2a | 9 | TTWDYYSSY | 2.11% | 5 | 3/1 | 3.11 | 0.34560 | 1/0 | 4.56 | 0.40160 |

[a] Length of H-CDR3 in amino acids.
[b] Revealed from nucleotide position 52 (codon 18) up to and including 312 (codon 104) according to IMGT nomenclature (http://imgt.cines.fr) (Lefranc et al., 1999).
[c] Mutation frequency (percent).
[d] Number of mutations.
[e] Observed R/S mutation ratio is the quotient of observed R to observed S mutations in H-FR of H-CDR3 regions. The actual number of R and S mutations are given for the observed R/S ratios.
[f] The theoretical expected R/S ratio is the quotient of total possible R and total possible S mutations in the germline gene, and is calculated according to Chang and Casali (1994).
[g] The possibility (P) that an excess or scarcity of replacement mutations in H-FR or H-CDR3 regions result solely by chance is negated by the significantly low probability values ($P < 0.05$) calculated according to the binomial distribution (Chang and Casali, 1994).
[h] Since the used constant region primer was located in the first exon of the C$\gamma$ regions, it is not possible to discriminate between IgG1 and IgG2a subclasses.
[i] Sequences found twice.
[j] Alternative family member are possible: for CM7: PC-22; for MZ17: PC-41; for MZ19: PC-26 or PC-33; for FO17: PC-14. The alternative family members give rise to same number of mutations and have no effect on significance levels of R/S ratios.
[k] The sequences MZ18 and FO26 are from clonally related B cells and form clone set #1 (see text).
[l] The sequences CM22 and MZ23 are from clonally related B cells and form clone set #2 (see text).

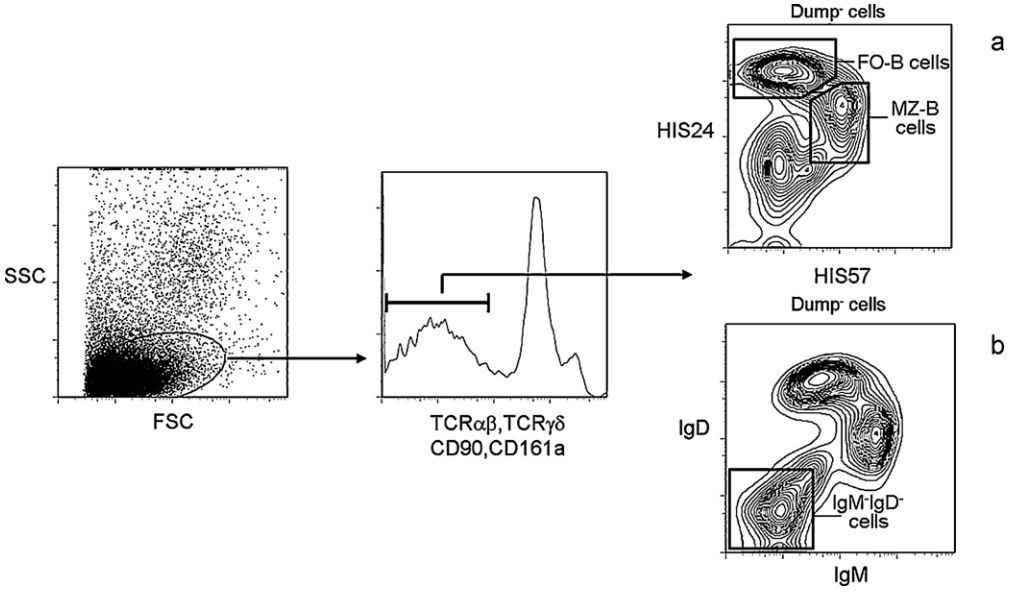


**Fig. 1.** Phenotype of MZ-B cells and FO-B cells in rat spleen. Spleen cell suspensions of rat spleen were stained with mAb directed against CD90, TCR$\alpha\beta$, TCR$\gamma\delta$ and CD161a to exclude immature B cells, T cells and NK cells from the analysis (Dump channel). These mAb's were combined with HIS24 (anti-CD45R) HIS57, a mAb directed to an unknown determinant on MZ-B cells (panel a) or combined with mAb directed against IgM and IgD (panel b). MZ-B cells are defined as Dump$^-$HIS24$^{lo}$HIS57$^{hi}$ cells, and FO-B cells as Dump$^-$HIS24$^{hi}$HIS57$^{-/low}$ cells. Classical, class-switched, memory cells are found among the Dump$^-$IgM$^-$IgD$^-$ fraction. SSC = side scatter, FSC = forward scatter.

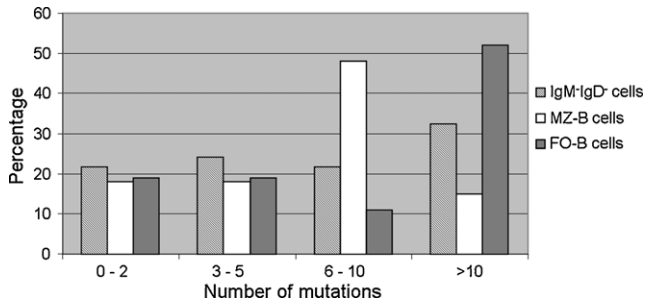


**Fig. 2.** IGHV genes encoding for IgG antibodies from B cells with a MZ-B or FO-B cell phenotype differ in mutation frequencies. PCR products from IGHV5-Cγ transcripts from FACS-purified MZ-B cells, FO-B cells and $IgM^{-}IgD^{-}$ cells were cloned and sequenced. Sequences were compared to known germline IGHV5 genes from PVG rats, and the numbers of mutations compared to these germline genes was calculated. Sequences divided into four groups based upon the number of mutations. Sequences with 0–2 mutations were considered to be unmutated, because we could not exclude the possibility that 1–2 mutations are the result of Taq errors. Class-switched B cells with a FO-B and MZ-B cell phenotype have a statistically different mutation profile (Fisher's exact test, $P = 0.004$).

exon of the Cγ regions. Since this part of the Cγ region is identical for both the Cγ1 and Cγ2a regions (Bruggemann, 1988), it was not possible to discriminate between these two IgG subclasses. Fig. 3 shows that the three B cell fractions express IGHV5-Cγ transcripts encoding for all IgG subclasses (IgG1/IgG2a, IgG2b and IgG2c). The usage of the various IgG subclasses appear, however, not to be statistically significant between the various B cell fractions (Fisher's exact test, $P = 0.127$).

### 3.4. The repertoire of class-switched IGHV region genes obtained from B cells with a MZ-B-cell or FO-B cell phenotype is highly similar.

There are no substantial differences in the usage of individual IGHV5 family members among the IGHV5-Cγ transcripts derived from the three B cells fractions (see Table 1). Some IGHV5 family-members are, however, more frequently expressed in the different fractions. For example, B cells with a MZ-B cell phenotype appear to use most frequently PC-5 (20%), PC-29 (16%) and PC-4 (13%) to encode for their IgG antibodies, whereas B cells with a FO-B cell phenotype use most frequently PC-5 (15%), PC-1 (15%) and PC-34 (15%). These differences are, however, not statistically significant (Fisher's exact test, $P = 0.60$).

Of the four IGHJ genes, the IGHJ2 gene was the most abundantly found among the IGHV-C$\gamma$ transcripts in all three B cell fractions, compared to the other IGHJ gene members. Approximately 50–60% of these IgG encoding transcripts appear to utilize the IGHJ2 gene, followed by IGHJ3 (20–30%) and the two other

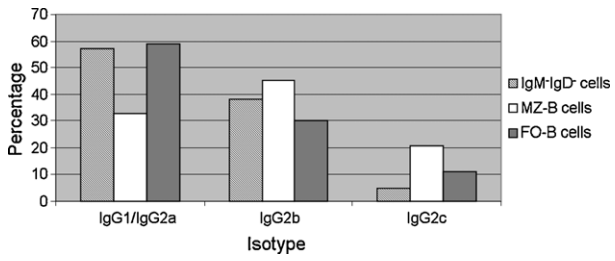


**Fig. 3.** Subclass distribution of IGHV5-Cγ transcripts derived from B cells with a MZ-B or FO-B cell phenotype are comparable. The subclass of IGHV5-Cγ transcripts obtained from purified MZ-B cells, FO-B cells and $IgM^{-}IgD^{-}$ cells was determined by comparing the part of the PCR-amplified Cγ region to known rat Cγ genes encoding for the various rat IgG subclasses. The sequence of this part of Cγ1 and Cγ2a regions are identical to each other and cannot be discriminated. The usage of the various IgG subclasses is similar in the three B cell subsets (Fisher's exact test, $P = 0.127$).

IGHJ genes. This biased usage of IGHJ2 genes is similar to the usage of IGHJ2 genes by mature, naive FO-B and MZ-B cells (Dammers et al., 2000). Also the utilization of IGHD genes by the IGHV5-Cγ transcripts is similar for all three B cell fractions (Fisher's exact test, $P = 0.794$). Of the known functional IGHD genes (Hendricks et al., 2010) the IGHD genes 1–6 and 1–7 appear to be preferentially used to encode for IgG antibodies in all three fractions.

We have previously shown that naive MZ-B cells and FO-B cells differ with respect to the length of H-CDR3 region (Dammers et al., 2000; Dammers and Kroese, 2005). This H-CDR3 region is the most important part of the H chain for antigen recognition. On average, the length of the H-CDR3 region of naive MZ-B cells is $10.9 \pm 2.8$ codons, which is 1.7 codons shorter than used by FO-B cells (Dammers et al., 2000). Here we show that the average length of the H-CDR3 regions of the sequenced IGHV5-C$\gamma$ transcripts derived from B cells with an MZ-B cell phenotype is $12.2 \pm 2.8$ codons and does not differ from the H-CDR3 codon length of encoding for IgG antibodies expressed by B cells with a FO-B cell phenotype ($12.6 \pm 3.4$ codons) (Mann–Whitney test, $P = 0.61$). In summary, we conclude that there are no major differences in the primary IGHVDJ repertoire between the IgG expressing B cells with a MZ-B cell phenotype and a FO-B cell phenotype.

### 3.5. IGHV-C$\gamma$ transcripts from B cells with an MZ-B cell phenotype show signs of antigen selection

High affinity antibodies which are generated during humoral immune responses, generally result in amino acid replacements in the CDR regions. Selection of antibodies that can bind with high affinity to a particular antigen takes place within the germinal center (GC) during the formation of memory B cells. The replacement over silent mutation (R/S) ratio of antigen-selected antibodies in the H-CDR regions is therefore higher than expected in case these mutations are randomly introduced. In contrast, selected antibodies favor relatively more silent mutations in the framework (FR) regions, resulting in lower R/S values than expected. In accordance to the method developed by Chang and Casali (1994) we calculated the binomial change for the R/S ratio of H-CDR and H-FR regions of the IGHV5-C$\gamma$ transcripts with more than 4 mutations. As we show in Table 1, approximately 40% of the IGHV5-C$\gamma$ sequences in all B cell fractions show signs of antigen selection, i.e. reveal a significantly higher R/S ratio for the H-CDR regions and/or a significantly lower R/S ratio for the H-FR regions than expected. Thus, there is evidence that both IgG antibodies produced by B cells with a MZ-B cell phenotype and FO-B cell phenotype are likely the result of antigen-selection.

### 3.6. Clonally related B cells are found in both MZ-B cell fraction and FO-B cell fractions

IGHV sequences with identical H-CDR3 regions obtained in completely separate PCR's are considered to be derived from clonally related cells. We found two sets of two clonally related sequences in one rat (rat #3). In this animal one of the MZ-B cell derived sequences (MZ18) was clonally related to a FO-B cell derived sequence (FO26) (set #1), and another MZ-B cell derived sequence (MZ23) was clonally related to an $IgM^{-}IgD^{-}$ B cell derived sequence (CM22) (set #2) (Table 1). The two members of set #1 have the same IgG subclass, whereas the two members of set #2 express different IgG subclasses (IgG2b and IgG2c, respectively). There are no shared mutations between the sequences of both members of set #1 and set #2, indicating that the two members of each clone may have developed independently from each other from one single naive B cell, upon antigenic stimulation.

## 4. Discussion

Most MZ-B cells present in rodent spleen show characteristic features of naive B cells, i.e. they express IgM molecules on their membrane of which the variable domains are encoded by unmutated V-region genes. A small fraction of $IgM^+$ MZ-B cells, however, carry mutated V-genes and are qualified as $IgM^+$ memory MZ-B cells. In this study we provide molecular evidence for the existence of a third type naturally occurring B cell population with a MZ-B cell phenotype in rat spleen, viz. class-switched memory MZ-B cells. Previous studies described the appearance of antigen-specific, class-switched MZ-B cells (and FO-B cells) after immunization of rats and mice with protein antigens (Gatto et al., 2004; Liu et al., 1988; Obukhanych and Nussenzweig, 2006), and that antigen (virus like particle)-specific MZ-B cells are encoded by somatically mutated IGHV genes (Bergqvist et al., 2010; Gatto et al., 2007). The work presented here, combines these data by directly showing the presence of somatically mutated IGHV5 transcripts encoding for IgG antibodies among the pool of purified rat MZ-B cells. These two characteristics, class-switching and somatic mutation, are hallmarks of classical, class-switched, memory B cells (Tangye and Tarlinton, 2009).

The developmental origin of class-switched memory MZ-B cells is not clear. Also the developmental relationship with their FO-B cell counterpart remains to be established. Memory cells are generally believed to be generated in GC's (for reviews see e.g. MacLennan, 1994; Manser, 2004). Proliferating GC B cells alter their BCR's by somatic hypermutation and class switching on their way to become memory cell. Toyama et al. (2002) have shown that Bcl6-deficient mice, which cannot develop GC's, are still able to generate class-switched memory cells after immunization with a foreign protein antigen, albeit that the V-genes of these memory cells are not mutated. Similarly, $CD40^{-/-}$ mice, which also cannot form germinal centers, are still able to generate intestinal IgA plasma cells whereas somatic hypermutations in these cells are absent (Bergqvist et al., 2010). These findings illustrate that somatic hypermutation, but not class switching, during the humoral immune response to exogenous antigens is indispensable of the GC microenvironment. The somatic mutations are, in principle, randomly introduced into the IGHV-genes (Winter and Gearhart, 1998). Mutated B cells are subsequently subjected to positive selection for B cells expressing BCR's that bind with high affinity to the inducing antigen. To this end, follicular dendritic cells uniquely located in the GC's present immune complexes to the proliferating B cells. Many of the IGHV5-C$\gamma$ transcripts from class-switched memory B cells with a MZ-B or FO-B cell phenotype show signs of selection. An appreciable proportion of the IGHV5-C$\gamma$ genes exhibit significantly more R mutations in H-CDR's and/or significantly fewer R mutations in the H-FR's, than expected from a random distribution of mutations. This indicates that after acquiring somatic mutations during proliferation the cells must have undergone some form of selection of their BCR's. Thus, the observation that the mutation patterns of IgG transcripts obtained from both B cells with a MZ-B or FO-B cell phenotype show signs of antigen-selection therefore favors the notion that both types of memory cells are probably GC derived.

It could be that class-switched memory cells with a MZ-B or FO-B cell phenotype are derived from antigen stimulated naive MZ-B cells and naive FO-B cells, respectively. Transfer experiments of purified B cell subsets into SCID mice have indeed revealed that both MZ-B cells and FO-B cells can generate GC's upon stimulation with T cell-dependent (protein) antigen, albeit that MZ-B cells appear to be far less effective (Song and Cerny, 2003). Furthermore, both anti-henn egg lysozyme (HEL) transgenic MZ-B cells and FO-B cells, transferred into wildtype recipients, are capable of generating a robust anti-HEL IgG1 response and forming GC's after immunization with a protein antigen (Phan et al., 2005). The entry of MZ-B cells into GC's in these experiments was, however, delayed. Although it thus seems possible that class switched MZ-B cells and FO-B cells are derived from their own naïve counterparts, we have some arguments to assume that naive B cells that give rise to class-switched memory B cells with a MZ-B or FO-B cell phenotype belong to the same B cell pool. First, even within the relatively small number of sequences analyzed in this study, we detected a set of sequences derived from clonally related cells (i.e. IGHV5 sequences with identical H-CDR3 regions, and same IGHV genes) with members in both the MZ-B and FO-B cell fraction (clone #1). Apparently, descendants of one and the same activated (naive) B cell can become either a class-switched B cell with a FO-B cell phenotype or a class switched MZ-B cell. Second, there are no differences in the primary, unmutated, H-chain repertoire between these two memory B cell subsets since usage of IGHV, IGHD and IGHJ gene segments is similar. Third, also the H-CDR3 lengths of the IGHV-C$\gamma$ transcripts between B cells with a FO-B or MZ-B cell phenotype are comparable: $12.2 \pm 2.8$ codons for class-switched MZ-B cells and $12.6 \pm 3.4$ codons for the class-switched FO-B cells, respectively. This is in contrast to naive, IgM expressing, MZ-B cells which have significantly shorter H-CDR3 regions compared to naive, IgM expressing, FO-B cells (Dammers et al., 2000; Makowska et al., 1999). Also Gatto et al. (2007) observed that the length of H-CDR3 regions expressed by virus-specific (possibly class-switched) MZ-B cells and FO B cells are comparable. Notably, the average H-CDR3 length of both class-switched B cell subsets is identical to the reported H-CDR3 length of naive FO-B cells (Dammers et al., 2000). Together, these observations may suggest that class-switched memory MZ-B cells are probably derived from naive FO-B cells and not from naive MZ-B cells, with their shorter H-CDR3 regions. In this context we like to mention that also naive FO-B cells can develop into naive MZ-B cells (Dammers et al., 1999; Guay et al., 2009; Srivastava et al., 2005; Vinuesa et al., 2003).

Although the repertoire of naturally occurring class-switched memory MZ-B cells is very similar to the repertoire of naturally occurring class-switched B cells with a FO-B cell phenotype, the numbers of mutations between the two subsets vary significantly. Class-switched MZ-B cells are enriched in the category of 7–10 mutations per sequence, whereas class-switched B cells with a FO-B cell phenotype have most sequences in the category of >10 mutations per sequence (Fig. 2). A similar mutational difference has been observed by Gatto et al. (2007) between antigen-specific B cells with a FO-B cell phenotype and a MZ-B cell phenotype after immunization with virus like particles. As shown by Bende et al. (2007) recirculating class-switched IgG memory cells in humans can participate in several successive GC reactions herewith acquiring possibly more mutations. *In vivo* intravital imaging in mice revealed that GC's are open structures and that high-affinity antigen-specific B cells can participate in pre-existing GC's (Schwickert et al., 2009). The difference in mutation frequency between class-switched memory B cells with a MZ-B or FO-B cell phenotype might therefore be explained by the migratory properties of MZ-B cells. It could be that similar to naive MZ-B cells also class-switched memory MZ-B cells are sessile cells. In contrast, FO-B cells recirculate between the various lymphoid organs, herewith giving them the opportunity to participate in multiple GC reactions, and acquiring more mutations.

An alternative explanation for the difference in mutation frequencies between class switched B cells with a MZ and FO-B cell phenotype might be that there are intrinsic differences between the precursor cells for the two different types of memory cells, such as levels in activation induced deaminase. This enzyme plays a critical role in both class switching and somatic hypermutation (Maul and Gearhart, 2010). As mentioned before, purified murine

MZ-B cells and FO-B cells can give rise to IgG expressing memory cells upon transfer into recipient animals followed by immunization (Phan et al., 2005; Song and Cerny, 2003). It could be that levels of activation induced deaminase in activated MZ-B cells are lower, compared to activated FO-B cells, possibly as a result of differences in the signalling requirements of the two subsets. Lower levels of this enzyme may consequently result in lower mutation frequencies. Preliminary data suggest that there are no significant differences in levels of mRNA encoding for activation induced deaminase in flow cytometry purified rat B cells with a MZ-B or FO-B cell phenotype (data not shown).

Since class-switched B cells with a FO-B cell phenotype carry more mutations in their IgG encoding transcripts than MZ-B cells, it is very unlikely that class-switched B cells with FO-B cell phenotype simply acquire a MZ-B cell phenotype, e.g. during differentiation towards plasma cells. This is supported by the observation that class-switched B cells with a MZ-B cell phenotype are absent from lymph nodes, whereas memory cells with a FO-B cell phenotype are present (Bergqvist et al., 2010; Gatto et al., 2007).

In summary, the present study shows that in addition to naive MZ-B cells and mutated $IgM^+$ memory MZ-B cells, also class-switched, somatically mutated cells with a MZ-B cell phenotype are present in rat spleen. It remains, however, to be formerly proven that these class-switched cells also reside in the anatomically defined splenic MZ. We speculate that these class-switched memory MZ-B cells are derived from naïve FO-B cells and are generated in GC's. The function of these classical, memory type MZ-B cells, is not known. Excitingly, Ettinger et al. (2007) demonstrated that human $IgG^+$ MZ (like) B cells can respond vigorously in an antigen- and T cell-independent fashion to the combination of IL-21 and B cell activating factor belonging to the TNF family (BAFF). Triggering by these cytokines results in the rapid differentiation of $IgG^+$ MZ-B cells into IgG-secreting plasma cells. Whether class-switched memory MZ-B cells in rodents respond similarly, remains to be seen. These B cells in human splenic MZ's are in close association with $CD4^+$ $T_H$-cells and dendritic cells that could potentially secrete IL-21 and BAFF, respectively (Ettinger et al., 2007). In rodents, however, T cells are absent from the MZ. Class-switched MZ-B cells may provide the immune system with a sessile pool of memory cells that reflects the antigenic experience of the animal. These cells may respond rapidly to the presence of blood-borne antigens by producing IgG antibodies in addition to naive MZ-B cell-derived IgM antibodies, herewith contributing to humoral immunity in this extremely dangerous situation.

## Conflict of interest

The authors report no conflict of interest.

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